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Induction of IgG2a Class Switching in B Cells by IL-27[1]

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IL-27 is a novel IL-12 family member that plays a role in the early regulation of Th1 initiation. However, its role in B cells remains unexplored. We here show a role for IL-27 in the induction of T-bet expression and regulation of Ig class switching in B cells. Expression of WSX-1, one subunit of IL-27R, was detected at the mRNA level in primary mouse spleen B cells, and stimulation of these B cells by IL-27 rapidly activated STAT1. IL-27 then induced T-bet expression and IgG2a, but not IgG1, class switching in B cells activated with anti-CD40 or LPS. In contrast, IL-27 inhibited IgG1 class switching induced by IL-4 in activated B cells. Similar induction of STAT1 activation, T-bet expression and IgG2a class switching was observed in IFN-γ-deficient B cells, but not in STAT1-deficient ones. The induction of IgG2a class switching was abolished in T-bet-deficient B cells activated with LPS. These results suggest that primary spleen B cells express functional IL-27R and that the stimulation of these B cells by IL-27 induces T-bet expression and IgG2a, but not IgG1, class switching in a STAT1-dependent but IFN-γ-independent manner. The IL-27-induced IgG2a class switching is highly dependent on T-bet in response to T-independent stimuli such as LPS. Thus, IL-27 may be a novel attractive candidate as a therapeutic agent against diseases such as allergic disorders by not only regulating Th1 differentiation but also directly acting on B cells and inducing IgG2a class switching. ***The Journal of Immunology*****, 2004, 173: 2479–2485.**

Recently, a novel member of the IL-12 family was identified and termed IL-27 (1). IL-27 is a heterodimeric cytokine that consists of a p40-related protein, EBV-induced gene 3 (EBI3),[3] and a newly discovered IL-12 p35-related protein, p28. IL-27 is produced by activated APCs such as macrophages and dendritic cells (DCs) and is able to induce proliferation of naive but not memory $CD4^+$ T cells, and synergizes with IL-12 in IFN-γ production by naive $CD4^+$ T cells (1). Previous studies on the mice lacking one subunit of IL-27R, type I cytokine receptor (2) or WSX-1 (3), revealed that IL-27 is required for the early initiation of Th1 responses, although it is not necessary for the maintenance of Th1 responses. Consistent with these results, it has been very recently reported that IL-27 induces T-bet and subsequent IL-12Rβ2 expression, which is a key Th1 commitment step where naive Th precursor cells commence differentiation into Th1 cells, by naive $CD4^+$ T cells through JAK1/STAT1 activation (4–7). However, the role for IL-27 in B cells remains unexplored yet.

Mature B lymphocytes undergo Ig class-switch recombination (CSR) by activation with CD40L or LPS to produce a single, specific Ig isotype including one of the IgG subclasses, IgA, and IgE (8). In this process, cytokines such as IL-4, IFN-γ, and TGF-β play particularly critical roles in B cell differentiation by directing the isotype specificity of CSR. For instance, IL-4 directs murine IgE and IgG1 isotype production by activating transcription factors such as STAT6, which bind to and transactivate the germline Cϵ and Cγ1 promoters (9). TGF-β selectively stimulates CSR to IgA (10) and IgG2b (11), whereas IFN-γ selectively stimulates it to IgG2a and under certain circumstances IgG3 (12). Recently, the T-box transcription factor T-bet has been shown to regulate IgG2a class switching in B cells (13, 14). T-bet was initially identified in T cells, where it essentially serves as a transcriptional regulator of Th1 differentiation by directly activating Th1-associated genetic programs and repressing Th2 cytokine production (15–17). T-bet-deficient MRL/*lpr* mice developed greatly impaired IgG2a titers and autoantibodies, and were unable to initiate CSR to IgG2a in response to LPS and IFN-γ in vitro (13). Moreover, T-bet was shown to be necessary for IgG2a class switching in response to T-independent signaling via LPS, but not T-dependent signaling through CD40 (14). More recently, it has been demonstrated that T-bet mRNA is induced by CpG treatment independent of IFN-γ and STAT1 signaling pathway in B cells and that the treatment of B cells with CpG inhibits IgE and IgG1 class switching induced by IL-4 and CD40 ligation (18).

Therefore, in the present study, we investigated a role for IL-27 in B cells. We have found that primary mouse spleen B cells express functional IL-27R and that IL-27 plays a role in the induction of T-bet expression and IgG2a class switching.

Materials and Methods

Reagents

Mouse rIL-4 and-CD28 (37.51), rIL-12, anti-FLAG (M2) and anti-actin, anti-CD3 (145-2C11) and anti-IL-2 (S4B6), anti-T-bet (4B10) and anti-STAT1, and anti-phosphotyrosine (pY)-STAT1 were purchased from BD

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[3] Abbreviations used in this paper: EBI3, EBV-induced gene 3; DC, dendritic cell; CSR, class-switch recombination; pY, phosphotyrosine; sc, single-chain; HPRT, hypoxanthine phosphoribosyl transferase.

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Biosciences (Franklin Lakes, NJ), R&D Systems (Minneapolis, MN), Sigma-Aldrich (St. Louis, MO), American Type Culture Collection (Manassas, VA), Santa Cruz Biotechnology (Santa Cruz, CA), and Cell Signaling Technology (Beverly, MA), respectively. Anti-CD40 (1C10) and LPS were purchased from R&D Systems and Sigma-Aldrich, respectively. Mouse rIFN-γ was kindly provided by Shionogi (Osaka, Japan).

Cell culture and mice

B cells and naive $CD4^+$ T cells were cultured in RPMI 1640 medium supplemented with 10% FBS and 50 μM 2-ME. HEK293-F cells were purchased from Invitrogen Life Technologies (Carlsbad, CA) and cultured in the serum-free medium (FreeStyle 293 Expression Medium, Invitrogen Life Technologies). BALB/c and C57BL/6 mice were purchased from Japan SLC (Hamamatsu, Japan). IFN-γ-deficient mice (19) of C57BL/6 background were established and maintained at our animal facility. $STAT1^{+/-}$ and $STAT1^{-/-}$ mice (20) of a mixed background of 129/Sv and C57BL/6 were kindly provided by Dr. R. D. Schreiber (Washington University, St. Louis, MO). T-bet-deficient mice (17) of BALB/c background were purchased from The Jackson Laboratory (Bar Harbor, ME). All mice were maintained under specific pathogen-free conditions and used in accordance with our Institutional Guidelines.

Preparation of purified rIL-27 protein

rIL-27 was prepared as a soluble tagged fusion protein by flexibly linking EBI3 to p28, which was reported to show a similar activity to the naive heterodimeric complex (1). Mouse IL-27 EBI3 and p28 cDNAs were isolated by RT-PCR using total RNA prepared from Con A-activated spleen cells. For preparation of single-chain (sc) IL-27 expression vector, fragments encoding the mature part of EBI3, followed by the $(Gly_4Ser)_3$ linker (21), and then by the mature coding sequence of p28 were generated by using standard PCR methods and cloned into p3xFLAG-CMV-9 (Sigma-Aldrich) vector, which has preprotrypsin signal peptide and 3xFLAG-epitope-tag sequences at N-terminal. HEK293-F cells were then transiently transfected with the expression vector by using 293fectin (Invitrogen Life Technologies) according to the manufacturer's instructions. After 3 days, culture supernatant was harvested and 3xFLAG-tagged rscIL-27 was purified by affinity chromatography using anti-FLAG (M2) affinity gel (Sigma-Aldrich). Protein concentration of purified 3xFLAG-tagged rscIL-27 was determined by titration in Western blotting with anti-FLAG (M2) using 3xFLAG-tagged rscIL-12 prepared similarly as described above as a standard. The concentration of 3xFLAG-tagged rscIL-12 was determined using rIL-12 as a standard in ELISA as described previously (22).

Preparation of purified B and T cells

Purified primary B cells ($B220^+$ cells >99%) were isolated from spleen by negative selection with a B cell isolation kit containing biotin-conjugated mAbs to CD43, CD4, and Ter-119 (Miltenyi Biotec, Bergisch Gladbach, Germany). Primary T cells were purified by passing spleen cells depleted of erythrocytes through nylon wool. The flow-through fraction was incubated with biotin-conjugated anti-CD8α, anti-B220, anti-Mac-1, anti-Ter-119, and anti-DX5, followed by incubation with anti-biotin magnetic beads (Miltenyi Biotec), and passed through a magnetic cell-sorting column (Miltenyi Biotec). The negative fraction containing purified $CD4^+$ T cells ($CD4^+$ cells >95%) was collected and then incubated with anti-CD62 ligand magnetic beads (Miltenyi Biotec). The positive fraction was collected and used as purified naive $CD4^+$ T cells ($CD62L^+$ cells >99%).

RT-PCR

Total RNA was extracted by using a guanidine thiocyanate procedure, cDNA was prepared using oligo(dT) primer and SuperScript RT (Invitrogen Life Technologies), and RT-PCR was performed using *Taq*DNA polymerase as described (22). Cycle conditions were 94°C for 40 s, 60°C for 20 s, and 72°C for 40 s. The following primers were used; WSX-1 sense primer, 5′-ACCCAAATGAAGCCAGACAC-3′; WSX-1 antisense primer, 5′-CACACAAGGTCTTGGGTCCT-3′; T-bet sense primer, 5′-TGCCTG CAGTGCTTCTAACA-3′; T-bet antisense primer, 5′-TGCCCCGCTTC CTCTCCAACCAA-3′. Primers used for IgG1 and IgG2a germline transcripts and hypoxanthine phosphoribosyl transferase (HPRT) were described (23, 24).

Western blotting

Cells were lysed in a lysis buffer containing protease inhibitors, and resultant cell lysates were separated on a SDS-PAGE under reducing conditions and transferred to polyvinylidene difluoride membrane (Millipore, Bedford, MA). The membrane was then blocked, probed with primary Ab and then with appropriate secondary Ab conjugated to HRP, and visualized with the ECL detection system (Amersham Pharmacia Biotech, Little Chalfont, U.K.) according to the manufacturer's instructions.

Ig and IFN-γ production assays

Quantitation of IgG1 and IgG2a isotypes in culture supernatants was performed in ELISA according to the manufacturer's instructions (Bethyl Laboratories, Montgomery, TX). For measurement of IFN-γ production, purified naive $CD4^+$ T cells (1×10^6 cells/ml) were stimulated with plate-coated anti-CD3 (2 μg/ml) and anti-CD28 (0.5 μg/ml) in the presence of rIL-12 and anti-IL-4 (10 μg/ml) for 48 h, and culture supernatants were collected and assayed for IFN-γ production by ELISA as described (25).

Results

Expression of functional IL-27R in primary spleen B cells

We first examined the expression of one subunit of IL-27R, WSX-1, in primary mouse spleen B cells at mRNA level by RT-PCR. The expression of WSX-1 mRNA in primary spleen B cells was detected even without the stimulation by anti-CD40 and was not greatly increased by the stimulation (Fig. 1*A*). The expression level of WSX-1 mRNA in B cells appeared to be similar to that in naive $CD4^+$ T cells activated with plate-coated anti-CD3. Because the other subunit(s) of IL-27R has not been identified yet and IL-27 was reported to activate STAT1 in naive $CD4^+$ T cells (4–7), we then examined whether IL-27 induces the tyrosine phosphorylation of STAT1 in primary spleen B cells without activation by anti-CD40 or LPS. Indeed, IL-27 induced the tyrosine phosphorylation of STAT1 in primary spleen B cells, which was detected by Western blotting 20 min after stimulation and decreased gradually thereafter, as IFN-γ did (Fig. 1*B*). These results suggest that primary spleen B cells express functional IL-27R and are able to mediate IL-27 signalings such as STAT1 activation.

Induction of T-bet expression and IgG2a class switching by IL-27 in primary spleen B cells

Because IL-27 was reported to induce T-bet expression in naive $CD4^+$ T cells activated with plate-coated anti-CD3 (4), we next examined whether IL-27 induced T-bet expression in B cells as well. Primary spleen B cells were stimulated with various amounts of IL-27 (0.1, 1, and 10 ng/ml) for 72 h and then subjected to

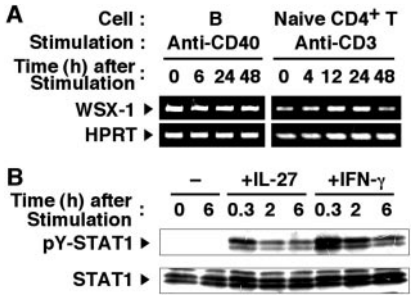

FIGURE 1. Expression of functional IL-27R in primary spleen B cells. *A*, Expression of WSX-1, one subunit of IL-27R, at mRNA level. Primary spleen B cells (1×10^6 cells/ml) obtained from wild-type BALB/c mice were stimulated by anti-CD40 (1 μg/ml), harvested after various times and subjected to RT-PCR analysis for WSX-1 mRNA expression. Naive $CD4^+$ T cells (1×10^6 cells/ml) were also stimulated by plate-coated anti-CD3 (2 μg/ml) in the presence of anti-IL-2 (100 μg/ml) and analyzed for WSX-1 mRNA expression by RT-PCR. *B*, Tyrosine phosphorylation of STAT1 detected by Western blotting. Primary spleen B cells (2×10^6 cells/ml) obtained from wild-type BALB/c mice were stimulated by IL-27 (5 ng/ml) or IFN-γ (200 ng/ml) as a positive control for various times and harvested. Then, total cell lysate was prepared and subjected to Western blotting using anti-pY-STAT1. For verification of the same protein expression level, the blot was reprobed with anti-total STAT1. Similar results were obtained in two to three independent experiments.

RT-PCR analyses for T-bet mRNA expression. The T-bet mRNA expression was enhanced by IL-27 in a dose-dependent manner as by IFN-γ (200 ng/ml; Fig. 2*A*). Similar induction of T-bet expression at protein level as well as at mRNA level was observed in spleen B cells activated by anti-CD40 or LPS (Fig. 2, *A* and *B*). Although protein recovery from spleen B cells stimulated by IL-27 without activation by anti-CD40 or LPS was much less than that with the activation, increased T-bet expression at protein level was also observed without the activation (data not shown). Because T-bet is important for IgG2a class switching in B cells (13, 14), we next examined whether IL-27 induces IgG2a class switching in activated B cells. Induction of IgG2a germline transcripts by IL-27 was detected by RT-PCR in spleen B cells activated with anti-CD40 or LPS for 72 h as that by IFN-γ (Fig. 2*A*). Next, activated spleen B cells were incubated in the presence and absence of various amounts of IL-27 for 6 days, and the culture supernatant was analyzed for IgG2a production in ELISA. Correlating with the induction of IgG2a germline transcripts, IgG2a production was markedly enhanced by the incubation with increasing amounts of IL-27 in B cells activated with LPS as with IFN-γ (Fig. 2*C*). These results suggest that IL-27 induces T-bet expression and IgG2a class switching in primary spleen B cells.

Inhibition of IL-4-induced IgG1 class switching by IL-27 in primary spleen B cells

IFN-γ is well known to inhibit IgG1 class switching as well (12) and T-bet was recently suggested to play a role in the inhibition of IgG1 class switching (13, 18). Therefore, we next examined the effect of IL-27 on IgG1 class switching. Primary spleen B cells were stimulated with various amounts of IL-27 (0.1, 1, and 10 ng/ml) and anti-CD40 or LPS in the presence or absence of IL-4 for 72 h and 6 days and subjected to RT-PCR, Western blotting, and ELISA, respectively, for analyses of T-bet expression and IgG1 class switching. IL-27 did not induce IgG1 class switching (Fig. 3, *A* and *C*) and the induction of T-bet expression by IL-27 was greatly diminished in the presence of IL-4 at both mRNA and protein levels (Fig. 3, *A* and *B*). In contrast, IL-27 partially but constantly inhibited IgG1 class switching induced by IL-4 in a dose-dependent manner (Fig. 3, *A* and *C*). These results suggest

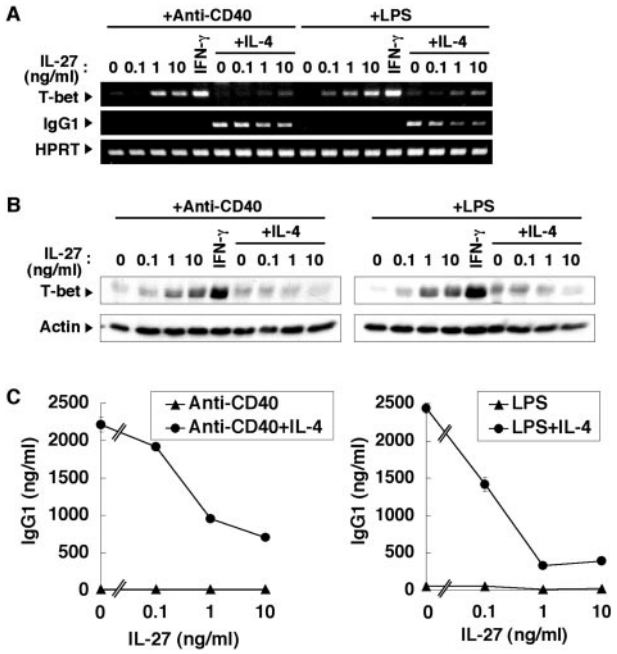

FIGURE 3. Inhibition of IL-4-induced IgG1 class switching by IL-27 in primary spleen B cells. *A*, Expression of T-bet and germline IgG1 mRNA detected by RT-PCR. Primary spleen B cells (5×10^5 cells/ml) obtained from wild-type BALB/c mice were stimulated by IL-27 (0.1, 1, and 10 ng/ml) and anti-CD40 (1 μg/ml) or LPS (10 μg/ml) in the presence or absence of IL-4 (10 ng/ml) for 72 h, and total RNA was prepared and subjected to RT-PCR analysis for T-bet, germline IgG1 and HPRT mRNA expression. (*B*) T-bet expression at protein level detected by Western blotting. The spleen B cells (2×10^6 cells/ml) were stimulated by IL-27 (0.1, 1, and 10 ng/ml) and anti-CD40 (1 μg/ml) or LPS (10 μg/ml) in the presence or absence of IL-4 (10 ng/ml) for 72 h, and total cell lysate was prepared and subjected to Western blotting using anti-T-bet and anti-actin. (*C*) IgG1 production measured by ELISA. The spleen B cells (5×10^5 cells/ml) were stimulated by IL-27 (0.1, 1, and 10 ng/ml) and anti-CD40 (1 μg/ml) or LPS (10 μg/ml) in the presence or absence of IL-4 (10 ng/ml) for 6 days, and the culture supernatant was analyzed for IgG2a production in ELISA in triplicate. Data are shown as the mean ± SD. IFN-γ (200 ng/ml) was used as a positive control in all these experiments. Similar results were obtained in three independent experiments.

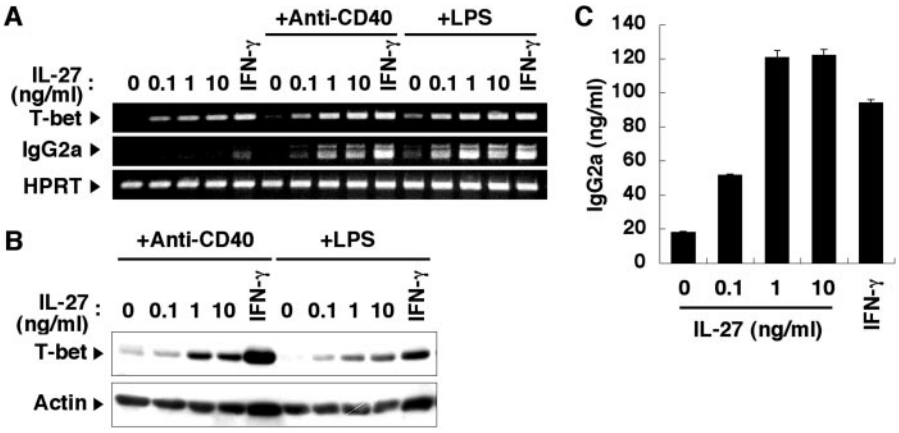

FIGURE 2. Induction of T-bet expression and IgG2a class switching by IL-27 in primary spleen B cells. *A*, Expression of T-bet and germline IgG2a mRNA detected by RT-PCR. Primary spleen B cells (5×10^5 cells/ml) obtained from wild-type BALB/c mice were stimulated by IL-27 (0.1, 1, and 10 ng/ml) in the presence and absence of anti-CD40 (1 μg/ml) or LPS (10 μg/ml) for 72 h, and total RNA was prepared and subjected to RT-PCR analysis for T-bet, germline IgG2a and HPRT mRNA expression. *B*, T-bet expression at protein level detected by Western blotting. The spleen B cells (2×10^6 cells/ml) were stimulated by IL-27 (0.1, 1, and 10 ng/ml) in the presence of anti-CD40 (1 μg/ml) or LPS (10 μg/ml) for 72 h, and total cell lysate was prepared and subjected to Western blotting using anti-T-bet and anti-actin. *C*, IgG2a production measured by ELISA. The spleen B cells (5×10^5 cells/ml) were stimulated by IL-27 (0.1, 1, and 10 ng/ml) in the presence of LPS (10 μg/ml) for 6 days, and the culture supernatant was analyzed for IgG2a production in ELISA in triplicate. Data are shown as the mean ± SD. IFN-γ (200 ng/ml) was used as a positive control in all these experiments. Similar results were obtained in four to five independent experiments.

that IL-27 does not induce IgG1 class switching but inhibits IL-4-induced IgG1 class switching. Thus, IL-27 may have a reciprocal relationship with IL-4 as IFN-γ has in B cells.

T-bet expression and IgG2a class switching induced by IL-27 in primary spleen B cells are independent of IFN-γ

IFN-γ also induces STAT1 activation, T-bet expression, and IgG2a class switching in B cells (Figs. 1 and 2). IL-27 induces IFN-γ production in activated naive $CD4^+$ T cells in collaboration with IL-12 (1, 4). Therefore, to exclude the possibility of potential involvement of IFN-γ in the effects of IL-27 as described above, we next used IFN-γ-deficient mice. First of all, we confirmed the inability of IFN-γ-deficient mice to produce IFN-γ using $CD4^+$ T cells activated by anti-CD3 and IL-12 (Fig. 4*A*). Then, we compared the STAT1 activation, T-bet expression at mRNA and protein levels, and IgG2a production between IFN-γ-deficient and wild-type mice. Even in IFN-γ-deficient spleen B cells, IL-27 induced tyrosine phosphorylation of STAT1 (Fig. 4*B*), T-bet expression at both mRNA and protein levels (Fig. 4, *C* and *D*), and IgG2a production (Fig. 4*E*) similar to those in wild-type spleen B cells. These results suggest that STAT1 activation, T-bet expression, and IgG2a class switching induced by IL-27 are independent of IFN-γ.

T-bet expression and IgG2a class switching induced by IL-27 in primary spleen B cells are dependent on STAT1

We have very recently found that STAT1 is indispensable for IL-27-mediated T-bet and IL-12Rβ2 expression but not for proliferation in naive $CD4^+$ T cells.[4] Therefore, we next investigated the role of STAT1 in IL-27-mediated T-bet expression and IgG2a class switching using primary spleen B cells obtained from $STAT1^{-/-}$ mice and $STAT1^{+/-}$ mice. We first confirmed the absence of STAT1 protein and tyrosine-phosphorylated STAT1 even after stimulation with IL-27 for 20 min in $STAT1^{-/-}$ spleen B cells but the presence of them in $STAT1^{+/-}$ spleen B cells (Fig. 5*A*). Then, these spleen B cells were activated with anti-CD40 or LPS in the presence of IL-27 for 72 h, harvested and subjected to RT-PCR and Western blotting to detect T-bet expression at mRNA and protein levels. Although IL-27 induced T-bet expression at both mRNA and protein levels in $STAT1^{+/-}$ spleen B cells, IL-27 barely induced it in $STAT1^{-/-}$ spleen B cells (Fig. 5, *B* and *C*). Next, the spleen B cells were activated with LPS in the presence of IL-27 for 6 days, and the culture supernatant was analyzed for IgG2a production in ELISA. Consistent with the T-bet expression, IgG2a production was greatly reduced in $STAT1^{-/-}$ spleen B cells as compared with that in $STAT1^{+/-}$ spleen B cells (Fig. 5*D*). These results suggest that STAT1 is important for T-bet expression and IgG2a class switching induced by IL-27 in spleen B cells.

IgG2a class switching induced by IL-27 in primary spleen B cells activated with LPS is dependent on T-bet

Because T-bet was recently demonstrated to be important for T-independent IgG2a class switching (14), we finally investigated the role of T-bet in IL-27-mediated IgG2a class switching using T-bet-deficient mice and wild-type BALB/c mice. T-bet-deficient spleen B cells activated with anti-CD40 or LPS were first confirmed not to express T-bet at all even after stimulation with IL-27 or IFN-γ for 72 h (Fig. 6*A*). However, expectedly, IL-27 still induced tyrosine phosphorylation of STAT1 in these B cells similar to that in wild-type B cells (Fig. 6*B*). Then, IL-27-induced IgG2a class switching was examined. The induction of IgG2a germline transcripts by IL-27 appeared to be reduced but was still observed in T-bet-deficient spleen B cells activated by anti-CD40 (Fig. 6*C*). In contrast, the induction of germline IgG2a transcripts and IgG2a production by IL-27 were almost completely abolished in T-bet-deficient spleen B cells activated by LPS (Fig. 6, *C* and *D*). These results suggest that T-bet is required for IL-27-induced IgG2a class

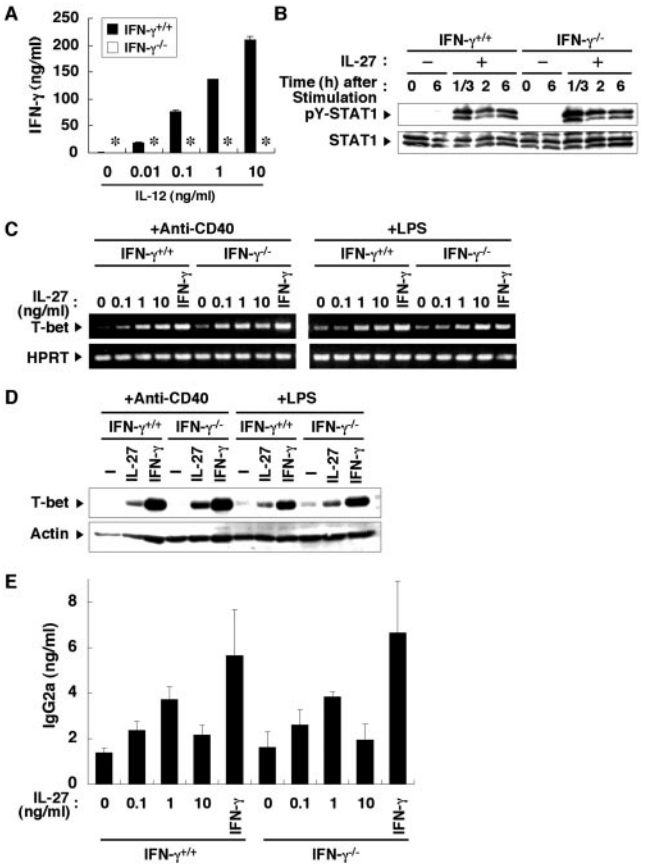

FIGURE 4. T-bet expression and IgG2a class switching induced by IL-27 in primary spleen B cells are independent of IFN-γ. *A*, IFN-γ production measured by ELISA. Naive $CD4^+$ T cells (1×10^6 cells/ml), obtained from IFN-γ-deficient mice or wild-type C57BL/6 mice, were stimulated by plate-coated anti-CD3 (2 μg/ml) and anti-CD28 (0.5 μg/ml) in the presence of IL-12 (0.01–10 ng/ml) and anti-IL-4 (10 μg/ml) for 48 h, and the culture supernatant was analyzed for IFN-γ production in ELISA in triplicate. Data are shown as the mean ± SD. ∗, <0.1 ng/ml. *B*, Tyrosine phosphorylation of STAT1 detected by Western blotting. Primary spleen B cells (2×10^6 cells/ml), obtained from IFN-γ-deficient mice or wild-type C57BL/6 mice, were stimulated by IL-27 (5 ng/ml) for various times, and total cell lysate was prepared and subjected to Western blotting using anti-pY-STAT1. For verification of the same protein expression level, the blot was reprobed with anti-total STAT1. *C*, T-bet mRNA expression detected by RT-PCR. The spleen B cells (5×10^5 cells/ml) were stimulated by IL-27 (0.1, 1, and 10 ng/ml) in the presence of anti-CD40 (1 μg/ml) or LPS (10 μg/ml) for 72 h, and total RNA was prepared and subjected to RT-PCR analysis for T-bet and HPRT mRNA expression. *D*, T-bet expression at protein level detected by Western blotting. The spleen B cells (2×10^6 cells/ml) were stimulated by IL-27 (5 ng/ml) in the presence of anti-CD40 (1 μg/ml) or LPS (10 μg/ml) for 72 h, and total cell lysate was prepared and subjected to Western blotting using anti-T-bet and anti-actin. *E*, IgG2a production measured by ELISA. The spleen B cells (5×10^5 cells/ml) were stimulated by IL-27 (0.1, 1, and 10 ng/ml) in the presence of LPS (10 μg/ml) for 6 days, and the culture supernatant was analyzed for IgG2a production in ELISA. Data are shown as the mean ± SD of three independent experiments. ∗, $p < 0.05$, compared with wild-type B cells. IFN-γ (200 ng/ml) was used as a positive control in all these experiments.

[4] S. Kamiya, T. Owaki, N. Morishima, F. Fukai, J. Mizuguchi, and T. Yoshimoto. An indispensable role for STAT1 in IL-27-induced T-bet expression but not proliferation of naive $CD4^+$ T cells. *Submitted for publication.*

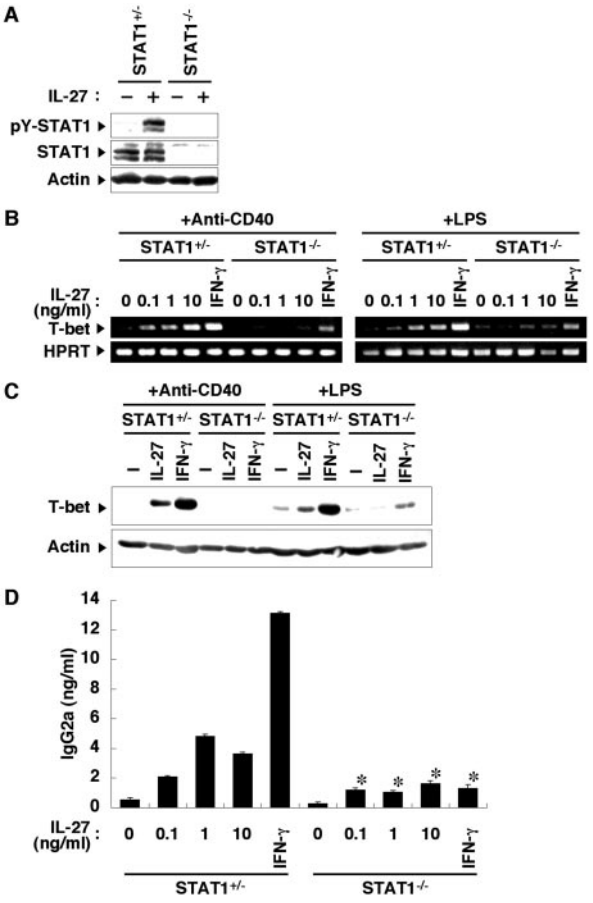

FIGURE 5. T-bet expression and IgG2a class switching induced by IL-27 in primary spleen B cells are dependent on STAT1. *A*, Tyrosine phosphorylation of STAT1 detected by Western blotting. Primary spleen B cells (2×10^6 cells/ml), obtained from STAT1$^{-/-}$ mice or STAT1$^{+/-}$ mice, were stimulated by IL-27 (5 ng/ml) for 20 min, and total cell lysate was prepared and subjected to Western blotting using anti-pY-STAT1 and anti-total STAT1. For verification of the same protein expression level, the blot was reprobed with anti-actin. *B*, T-bet mRNA expression detected by RT-PCR. The spleen B cells (5×10^5 cells/ml) were stimulated by IL-27 (0.1, 1, and 10 ng/ml) in the presence of anti-CD40 (1 μg/ml) or LPS (10 μg/ml) for 72 h, and total RNA was prepared and subjected to RT-PCR analysis for T-bet and HPRT mRNA expression. *C*, T-bet expression at protein level detected by Western blotting. The spleen B cells (2×10^6 cells/ml) were stimulated by IL-27 (5 ng/ml) in the presence of anti-CD40 (1 μg/ml) or LPS (10 μg/ml) for 72 h, and total cell lysate was prepared and subjected to Western blotting using anti-T-bet and anti-actin. *D*, IgG2a production measured by ELISA. The spleen B cells (5×10^5 cells/ml) were stimulated by IL-27 (0.1, 1, and 10 ng/ml) in the presence of LPS (10 μg/ml) for 6 days, and the culture supernatant was analyzed for IgG2a production in ELISA in triplicate. Data are shown as the mean ± SD. *, $p < 0.05$, compared with wild-type B cells. IFN-γ (200 ng/ml) was used as a positive control in all these experiments. Similar results were obtained in three independent experiments.

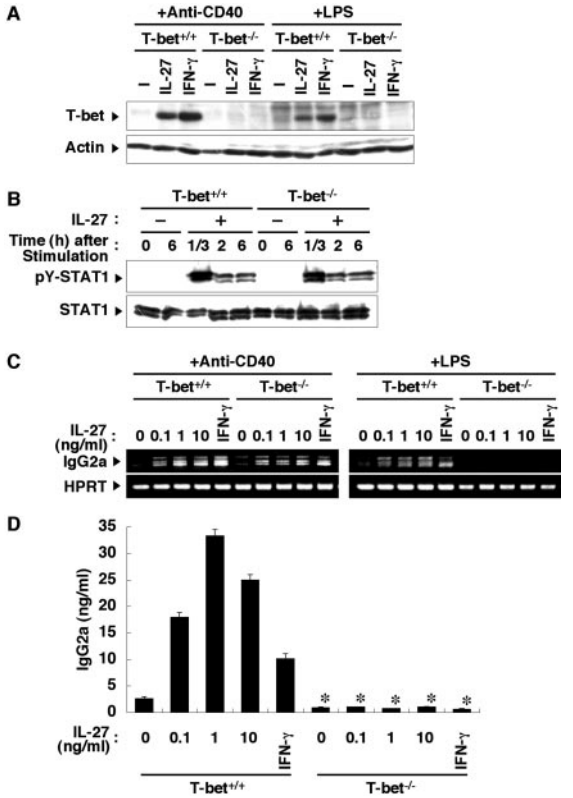

FIGURE 6. IgG2a class switching induced by IL-27 in primary spleen B cells activated with LPS is dependent on T-bet. *A*, T-bet expression at protein level detected by Western blotting. Primary spleen B cells (2×10^6 cells/ml), obtained from T-bet-deficient mice or wild-type BALB/c mice, were stimulated by IL-27 (5 ng/ml) in the presence of anti-CD40 (1 μg/ml) or LPS (10 μg/ml) for 72 h, and total cell lysate was prepared and subjected to Western blotting using anti-T-bet and anti-actin. *B*, Tyrosine phosphorylation of STAT1 detected by Western blotting. The spleen B cells (2×10^6 cells/ml) were stimulated by IL-27 (5 ng/ml) for various times, and total cell lysate was prepared and subjected to Western blotting using anti-pY-STAT1. For verification of the same protein expression level, the blot was reprobed with anti-total STAT1. *C*, Germline IgG2a mRNA expression detected by RT-PCR. The spleen B cells (5×10^5 cells/ml) were stimulated by IL-27 (0.1, 1, and 10 ng/ml) in the presence of anti-CD40 (1 μg/ml) or LPS (10 μg/ml) for 72 h, and total RNA was prepared and subjected to RT-PCR analysis for IgG2a and HPRT mRNA expression. *D*, IgG2a production measured by ELISA. The spleen B cells (5×10^5 cells/ml) were stimulated by IL-27 (0.1, 1, and 10 ng/ml) in the presence of LPS (10 μg/ml) for 6 days, and the culture supernatant was analyzed for IgG2a production in ELISA in triplicate. IFN-γ (200 ng/ml) was used as a positive control in all these experiments. Data are shown as the mean ± SD. *, $p < 0.05$, compared with wild-type B cells. Similar results were obtained in three to five independent experiments.

switching in B cells in response to T-independent stimuli such as LPS, but does not appear to be essential for it in T-dependent stimuli such as anti-CD40.

Discussion

In the present study, we have elucidated a role for IL-27 in B cells. Primary mouse spleen B cells were found to express functional IL-27R (Fig. 1), and IL-27 induced T-bet expression and IgG2a, but not IgG1, class switching in these B cells activated with anti-CD40 or LPS (Figs. 2 and 3). In contrast, IL-27 inhibited IgG1 class switching induced by IL-4 in activated B cells (Fig. 3). Similar induction of tyrosine phosphorylation of STAT1, T-bet expression and IgG2a class switching by IL-27 was observed in IFN-γ-deficient spleen B cells (Fig. 4), but not in STAT1-deficient ones (Fig. 5). The induction of IgG2a class switching was abolished in T-bet-deficient spleen B cells activated with LPS (Fig. 6). These results suggest that IL-27 play a role in the induction of T-bet expression and IgG2a, but not IgG1, class switching in B cells. Regarding the role of T-bet in the regulation of IgG2a class switching induced by IFN-γ, T-bet was reported to be necessary for IgG2a class switching in response to T-independent signaling via LPS, but not necessary for it in response to T-dependent signaling through CD40 (13, 14). Although IL-27 appears to have the similar

property to IFN-γ, further studies are necessary to clarify the regulation of IgG2a class switching by IL-27 in response to T-dependent signaling through CD40.

The IgG2a Ig subclass plays a critical role in the pathogenesis of humoral autoimmunity and protection against pathogens. The IgG2a is often pathogenic in autoantibody-mediated diseases like lupus, particularly in relationship to IFN-γ production (26–28). Antiviral Abs elicited by infection with a variety of common viruses are largely restricted to the IgG2a isotype (29). However, molecular mechanisms underlying the regulation of IgG class switching to IgG2a have not been fully elucidated. IFN-γ was previously demonstrated to selectively stimulate IgG2a class switching in B cells (12). A number of reports have confirmed the critical role of this cytokine in both in vitro and in vivo IgG2a responses, including after viral and parasitic infections (30–34). However, several reports have also demonstrated a partial IFN-γ independence of specific or total IgG2a responses in the course of infections, suggesting that there may be different pathways leading to the secretion of IgG2a isotype (31, 35–38). Thus, it has been speculated that other cytokines may also regulate the IgG2a class switching in some circumstances. IL-12 is produced after viral infection and increases IgG2a secretion but in an IFN-γ-dependent manner (39–41). IFN-α is largely produced after viral infection from plasmacytoid DC (42) and has been demonstrated to induce Ig class switching to IgG2a in an IFN-γ-independent fashion (13, 37, 43). However, in the infection with a certain virus such as influenza A virus, mice lacking both IFN-αβR and IFN-γR were demonstrated to still produce comparable levels of virus-specific IgG2a Abs to wild-type mice (44), suggesting that cytokines other than IFN-γ and IFN-α may possibly regulate IgG2a class switching in a certain infection. Because IL-27 is produced by macrophages and DC (1) and able to induce IgG2a class switching in activated B cells as shown in the present study, IL-27 may regulate the IgG2a class switching in the infected mice lacking both IFN-αβR and IFN-γR. However, the role of endogenous IL-27 in the regulation of IgG2a class switching under physiological conditions remains to be clarified.

It has been very recently reported that IL-27 activates JAK1, STAT1, -3, and -5 in naive $CD4^+$ T cells (4–7). We have also found that IL-27 activates JAK1, -2, tyrosine kinase 2, STAT1, -2, -3, and -5 in naive $CD4^+$ T cells and that STAT1 plays an indispensable role in IL-27-induced T-bet and IL-12Rβ2 expression but not proliferation of naive $CD4^+$ T cells (manuscript submitted). As JAK/STAT signaling molecules, IFN-αβ use JAK1, tyrosine kinase 2, STAT1, and -2, and IFN-γ uses JAK1, -2, and STAT1 (45–51), notably, whose patterns are similar to those activated by IL-27. If two cytokines activate the same JAK/STAT signaling molecules, it is reasonable to expect similar biological actions between them. Indeed, IL-27 has been very recently shown to induce T-bet and IL-12Rβ2 expression in naive $CD4^+$ T cells (4, 5) as IFN-γ does (52, 53). In addition, the present study revealed the roles of IL-27 in B cells including induction of STAT1 activation, T-bet expression and IgG2a class switching presumably similar to IFN-γ, further supporting the notion that IL-27 may have functional similarities to IFN-γ. If IL-27 could mimic IFN-αβγ, then the expression pattern of their receptors will be important. Although IFN-αβγRs are ubiquitously expressed on various tissues and cell types, the expression of one subunit of IL-27R, type I cytokine receptor, was reported to be detected in all cell types examined including $CD4^+$ and $CD8^+$ T cells, B cells, NK cells, and macrophages with highest levels in $CD4^+$ T cells and NK cells (2), but the other IL-27R subunit(s) remains to be identified.

Taken together, the present study suggests that IL-27 plays a role in the induction of IgG2 class switching in B cells, as well as in the early regulation of Th1 initiation in naive $CD4^+$ T cells through mainly the induction of T-bet expression. Thus, IL-27 may be a novel attractive candidate as a therapeutic agent against diseases such as allergic disorders by not only regulating Th1 differentiation but also directly acting on B cells and inducing IgG2a class switching.

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